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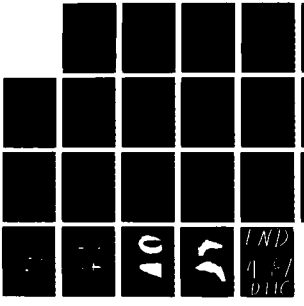

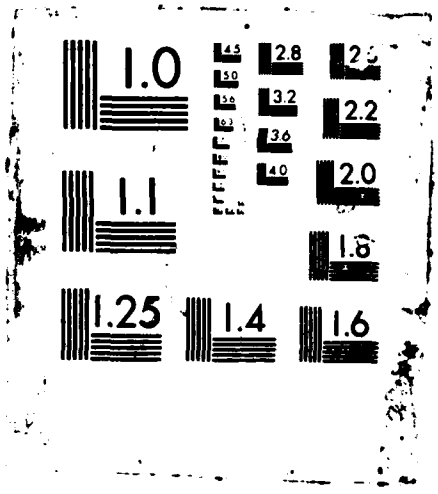
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DISTRIBUTION AND CHARACTERIZATION OF ANTIGENS FOUND IN SUBCELLULAR FRACTIONS OF AFRICAN TRYPANOSOMES

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An investigation of the distribution of antigen in the African trypanosome, Trypanosoma rhodesiense, has identified two principal antigenic sites, the surface and flaggellar pocket membranes (FPM). The latter, which was the principal subject of this study, was revealed to possess 2-3 principal glycoprotein antigens which are catinately membrane associated, exhibit no cross reaction with variable surface antigen (VSA) and are able to confer protection in mice against challenge infections using a limited T. rhodesiense scrodeme. The two principal FPM antigen have M_r 60-65x10^3, are selectively released by both phospholipase A_2 and sphingomyelinase and have been deduced to be oriented toward the lumen of the flagellar pocket and then accesible to antibody.

During the course of this study ancillary information relating to the subcellular organization of T. rhodesiense was obtained. This includes details of the disposition of enzymes within the glycosome, particularly the membrane association of adenylalle kinase and it's →

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A

19. ABSTRACT

strict and absolute requirement for phosphatidyl choline. In a search for surface membrane marker enzymes, an aid to cell fractionation procedures, a high affinity Ca^{2+} ATPase was discovered which is unusual in having no Mg^{2+} requirement. In addition a tartrateinsensitive, heat stable acid phosphatan was found which showed preferencial though not exclusive enrichment in surface membrane.

B

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FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

A. INTRODUCTION

The principal objectives of this project have been to identify the subcellular localization of antigenic components of bloodstream forms of the African trypanosome, Trypanosoma rhodesiense. In studying trypanosomal antigens, attention has been almost exclusively directed toward elucidating the structure and genetic control of the variant surface antigen (1). There are and continue to be valid reasons for investigating the localization and nature of other antigenic sites, not only for potential immunoprophylaxis, but also for improving diagnostic procedures.

Before any detailed investigation of the subcellular localization of antigens could be attempted, it was necessary to develop a reproducible procedure for cell fractionation enabling the unambiguous identification of cell fractions. During the course of this phase of the investigation considerable information was obtained, which whilst not directly germaine to the immediate goal of the project, has enabled a far greater understanding of various aspects of the functional organization of African trypanosomes. Thus details concerning the organization of the glycosome and the enzymic components of both the flagella pocket and surface membrane have been obtained. One aspect of our studies of the trypanosome surface membrane, namely the identification of a high affinity Ca-ATPase (2) now assumes added significance in view of the probable involvement of Ca^{2+} regulated process in VSA release (3).

Through the use of various immunoelectrophoretic techniques it has been possible to identify two principal subcellular antigenic sites, one of these being the surface membrane and the other the flagella pocket membrane. It is with this latter membrane site that this project has been mainly concerned. The results obtained describe the major properties of these flagella pocket membrane associated antigens and reveal amongst other feature that they exhibit no cross reaction with VSA, are intimately member associated and confer protection in mice against challenge infections with a limited

T. rhodesiense serodeme.

Apart from any obvious potential in vaccine development, the use of anti-FPM antibodies as a means of targeting liposome encapsulated trypanocidal drugs would seem to be an additional area worthy of investigation. Further work is most urgently required however, to further elucidate the interaction of host protective mechanisms with the flagellar pocket membrane.

B. METHODS

Most of the procedures used in this project have been cited or described in detail in previous publications (4,5,6). Summarized below are items of background information, not included in these publications, that describe procedures that either yielded negative though useful information, or were used to justify a given experimental approach.

1) Maintainence and isolation of bloodstream forms of Trypanosoms rhodesiense

The Wellcome CT strain of T. rhodesiense was routinely maintained in inbred Swiss mice at the Rane Laboratory, University of Miami. Parasites were recovered from blood, drawn by cardiac puncture, from Sprague Dawley rats infected intraperitoneal 3 days previously. Trypanosome recovery utilized the DEAE-cellulose procedure of Lanham (7), the following factors contributing greatly to successful parasite isolation. It was found extremely important to use coarse particle size DEAE cellulose (Sigma) that had been recycled (in INNaOH/0.5M NaCl then in INHCl) and extensively washed of fines, in order to obtain an acceptable flow rate. It was also essential to subject the blood to a 10 minute centrifugation, prior to ion-exchange purification, at 1500 r.p.m. (SC-34 rotor) and use the very pronounced buffy coat, where most of the parasites were found, for DEAE cellulose purification. We routinely use two 12x5 cm Buchner funnels through each of which is passed 30 - 40 ml buffy coat (from 120-150 mls blood). Parasites are washed through the cellulose using 300 - 400 ml Tris buffered glucose (7) and after centrifuga-

tion at 2500 r.p.m. (HS 4 rotor), twice washed with buffered sucrose (2mM KCl, 1.5 mM EDTA, 5mM HEPES, 250mM sucrose, pH 7.2).

2) Cell Breakage. The procedure used for cell breakage utilizing glass beads as an abrasive agent has been described in detail (4,5). It is worth stating once more that trypanosomes are unusually difficult cells to disrupt, due to the presence of a sub-pellicular microtubular network that is attached to the surface membrane. For this reason the more usual and less drastic methods, such as Dounce or Potter-Elvhem homogenizers are totally ineffective.

3) Cell Fractionation

a) Differential Centrifugation. Before establishing a suitable protocol for the initial fractionation of the trypanosome homogenate different times and rates of centrifugation were tested. Of these, the procedure now in current use is described in a previous publication (5) and yields two subcellular particle fractions Pa and Pb which have been employed for further fractionation and for the preparation of anti-sera.

b) Assay of Potential Marker Enzymes

In order to characterize the subcellular fractions obtained, a number of enzyme activities were investigated as markers. The choice of enzymes was based upon their known association in both mammalian cells in general and those available from previous studies of trypanosomes.

i) Surface Membrane. Initially three potential surface membrane enzymes were investigated: 5'-mononucleotidase (8) Na+, K+ stimulated Mg-ATPase (9) and alkaline p-nitrophenyl-phosphatase (10). In later studies two other enzyme activities were investigated, 3'-mononucleotidase, reported to be a surface membrane enzyme in Leishmania donovani was assayed as described (5), as well as a high affinity Ca-ATPase which we have described (2).

Less specific acid phosphatases were also considered as potential surface membrane

markers since previous investigation of other kinetoplastid flagellates (11) had indicated such a localization. The methods used, as well as results obtained are detailed in a recent report (12).

ii) Lysosomal Hydrolases. Proteolytic activity toward both denatured hemoglobin and a range of artificial low molecular weight substrates was assessed. Assays using the hemoglobin substrate were as previously described (13). The following artificial substrates were used as referenced: n-t Boc-alanine-2-nitrophenol (14) and succ-$(Ala)_3$-p-nitroanilide (15) both used as elastase substrates; leucine-2-naphthylamide (arylamidase 5), leucinamide (leucine aminopeptidase 5), cbz-glutamyl-tyrosine (carboxy-peptidase 5,13). Proteolytic activity was also assayed using the following dye-conjugated proteins: azocasein, azocoll and Congo red-elastin.

A number of organic monophosphates were used to determine the range of phosphatase activities present, as previously (12) described. Glucose-6-phosphate at 15 mM was assayed using 60 mM cacodylate, pH 6.0 $\pm$ 10 mM Na+K+ tartrate and 0.1% Triton X-100. After a 20-30 min incubation at 30°C released PO_4 was determined as above.

Attempts to measure glycosidase activities against a range of 4-methylumbelliferyl galactose, xylose and fucose substrates followed established methods.

iii) Mitochondrial Enzymes. An effort was made to measure oligomycin sensitive Mg-ATPase (16) one of the few enzymes claimed to be still active in the highly repressed mitochondrion of bloodstream African trypanosomes (17). The other potential mitochondrial enzyme assayed was a dichlorophenolindophenol (DCPID) linked α-glycerophosphate dehydrogenase, part of the α-glycerophosphate oxidase system, using a previously referenced procedure (4).

Adenylate kinase was also assayed and was of initial interest as a potential mitochondrial marker but proved to be mostly associated with glycosomes (4).

iv) Glycosomal Enzymes. For most purposes NAD+ linked α-glycerophosphate

dehydrogenase was used to monitor the distribution of glycosomes (5). In earlier investigations both phosphofructokinase and hexokinase were also assayed (4) as referenced.

v) Miscellaneous Enzymes. An attempt was made to establish the presence of authentic glucose-6-phosphatase (12) and diaphorase activities as markers for endoplasmic reticulum.

Assays of nucleoside diphosphatase and thiamine pyrophosphatase which were potential golgi body/e.r. markers followed established methods (18).

Covalent surface labelling of intact trypanosomes. Since there proved to be a paucity of authentic surface membrane enzymes, especially in the earlier stages of the investigation, intact trypanosomes were surface labelled using fluorescamine (Fluram, Roche Diagnostics) either free (5) or complexed to β-cyclodextrin (6,19). Relative fluorescence was then determined in the various subcellular fractions obtained.

4) Electrophoretic Procedures for Antigenic Analysis

a) Methods using agarose incorporated antibodies. Fused rocket immunoelectrophoresis (FRI) and crossed immunoelectrophoresis were performed as described (5,6) as was the isolation of IgG, for incorporation into gels, and the detergent extraction of subcellular fractions. Other treatments of specific subcellular fractions i.e. phospholipase or limited trypsinolysis have been described (20). In some instances, see above references, an intervening lectin affinity gel was spaced between the sample and antibody gel.

b) SDS-Polyacrylamide gel electrophoresis and electroblotting. The following methods of this aspect of the project are detailed in reference 20: sample preparation; separation of FPM proteins by polyacrylamide gel electrophoresis in the presence of SDS; staining of gels; immunoblotting procedures and post transfer treatment of blots to reveal proteins having antigenic and carbohydrate groups.

5) The subcellular distribution of protective antigens

Initial experiments (20) used 1.5 ml of each gradient fraction, obtained after isopycnic gradient centrifugation of either fractions P_a or P_b, to which was added 0.75 ml Freunds complete adjuvant (Hoechst-Calbiochem). For individual gradient fractions five mice were used each animal receiving 0.4 ml antigen injected subcutaneously into the shoulder area. Antigen was administered on days 0, 5, 14, 20 and 25 as was adjuvant mixed with 250 mM sucrose (1:2 v/v) to a series of 2 controls for each gradient fraction. A total of 20-24 gradient fraction were obtained after isopycnic centrifugation of fractions P_a, P_b requiring, together with appropriate controls 280 - 336 animals for one experiment. After a 12 day period following the final injection of antigen, mice were challenged with 1,000 trypanosomes, contained in 0.2 ml saline diluted injected mouse blood, administered intraperitoneally. This number of parasites was established as the MLD for the CT strain of T. rhodesiense currently maintained in the Rane Laboratory (U.M.).

Most recently FPM samples have been isolated from T. rhodesiense (CT strain, from the Rane Laboratory) by centrifuging fraction P_b on a discontinuous sucrose gradient as described. These preparations have been sent to WRAIR and under the direction of Dr. John G. Olenick have been administered to groups of inbred Swiss mice to study protection against cloned isolates of T. rhodesiense -- (V_{13}, V_{10} and CP3B4).

C) RESULTS

1) Enzyme distribution in subcellular fractions

The initial goal of this project was to establish a range of enzyme markers that could be used to identify subcellular fractions obtained during antigenic analysis. Reference to Table 1 shows summarized the subcellular distribution of various enzyme activities found to be present in bloodstream T. rhodesiense. The information is based for the most part on published studies as well as those results discussed in the manu-

script that forms appendix 1 at the end of this report.

Those enzyme activities exhibiting sufficient exclusivity in their localization to be used as markers are shown with an asterisk. Routinely the following were used as marker enzymes: FPM -- acid phosphatase (β-glycerophosphate), arylamidase; promitochondria -- DCPIP linked α-glycerophosphate dehydrogenase; lysosome -- proteinase (denatured hemoglobin) or carboxypeptidase (cbz-glu-tyr) and glycosomes -- NAD+ linked α-glycerophosphate dehydrogenase. For surface membrane the distribution of relative fluorescence, after fluorescamine labelling, was followed. This also labelled flagella pocket membrane, but this particle population exhibits a much lower equilibrium density than surface membrane. Relative fluorescence was also used in preference to assaying the high affinity Ca^{2+} -- ATPase, which is a more exclusive surface membrane constituent but not as convenient to measure. Although the level of relative fluorescence found in the cell sap ($\bar{}$15%) was above the 5% usually accepted for other surface markers, there was no evidence of significant labelling of intracellular sites. Reference to frequent experiments where this labelling procedure has been used for T. rhodesiense reveals no specific labelling of subcellular particles occuring in mid density ranges -- lysosomes or mitochondria. In addition a high proportion of the label in the final cell sap fraction is almost certainly derived from the contents of the flagellar pocket and in particular released VSA. The ready release of VSA has been documented in a number of previous investigations of T. brucei (3).

Reference to table 1 discloses the presence of certain other enzyme activities associated with these cell fractions as well as the absence of enzymes commonly used as marker for other eukaryotic cells, particularly of mammalian origin. Thus in keeping with the results of Steiger, et al. (21) with T. brucei we have been unable to authenticate the presence of a specific Na^+K^+Mg-ATPase and 5'-mononucleotidase as surface membrane components (5) or glucose-6-phosphatase as a microsomal marker. The surface

membrane does indeed possess Mg-ATPase activity but it is not influenced by Na, K^+ or Ca^{2+}, nor it susceptible to inhibition by oubain. In addition the 5'-mononucleotidase, as well as the glucose-6-phosphatase, that were present in a T. brucei surface membrane preparation (22) are almost certainly due to the tartrate resistant acid phosphatase we describe in T. rhodesiense surface membrane (12). The other surface membrane enzyme activity we have found, though not studied in any detail, is a leucine aminopeptidase like activity, though it differs from other similar surface membrane aminopeptidases in not being effected by divalent cations.

In L. donovani an unusual 3'-mononucleotidase has been found as a surface membrane component, rather then the usual 5'-mononucleotidase (11). Our studies revealed a similar enzyme in T. rhodesiense, also exhibiting a strict requirement for Co^{2+}, but the activity seems to be predominantly associated with FPM rather than surface membrane.

In any cell fractionation study of kinetoplastid flagellates it is necessary to appreciate the unusually high equilibrium density of surface membrane, due to the presence of attached microtubules. This has been found in investigations of Leptomonas collosoma (23), L. donovani (24) and T. brucei (22). In some studies (i.e. 21) surface membrane appears to have a much lower equilibrium density, but this is probably due to the detachment of microtubules during cell breakage having been found in T. brucei at least, to be clearly influenced by the buffer used (25).

The glycosomes equilibrated very near to surface membrane, but the enzymes associated with these organelles characteristicly exhibited much less tailing toward lower density regions of the gradient than surface membrane enzymes. One very interesting finding is the association of adenylate kinase, normally found in the mitochondrial inter-membrane space, with the glycosomal membrane (4,26). Most unique is the absolute dependence on phosphatidyl choline which is indicative of the intimate nature of the membrane association (26).

The flagellar pocket membrane (FPM), as had been established in previous investigations (5,21) possessed much of the acid phosphatase activity. The present study has revealed however (12), that in contrast to the enzyme present in surface membrane, the FPM acid phosphatase more closely resembles the typical "lysosomal" enzyme being much more sensitive to tartrate inhibition. Many other hydrolases are found associated with the FPM, particularly a range of peptidase activities. As has been suggested previously this probably reflects the importance of the flagella pocket as an uptake site for various nutrients (5). Possibly this is due to the presence of VSG on the surface of the parasite. It is known that in procyclic T. brucei, where surface coat is absent, there is much less acid phosphatase activity associated with the flagellar pocket (25). The considerable evidence of the importance of the flagellar pocket membrane as an uptake site is of course relevant to subsequent findings, reported below, on the protective nature of FPM antigens.

The most reliable mitochrondrial marker in the present investigation was found to be the DCPIP-linked α-glycerophosphate dehydrogenase. A small amount of activity was always found to co-sediment with surface membrane, but it is probably due to agglutinated mitochrondrial fragments. The large convoluted mitochondrion of kinetoplastids is easily damaged during the relatively harsh procedures required for cell disruption. As commented on previously (2,5) there is no oligomycin sensitive Mg-ATPase in bloodstream T. rhodesiense in contrast to bloodstream T. brucei (17). This loss of oligomycin sensitivity correlates with the apparent loss of nucleotide specificity, there being both ATPase and GTPase activities that co-sediment with the mitochondria as deduced from the distribution of DCPIP linked α-glycerophosphate dehydrogenase. A loss of nucleotide specificity is known to occur on removal of the oligomycin sensitivity-conferring protein form sub-mitochrondrial particles (27).

The distribution of lysosome like particles was routinely followed using proteinase

activity. A range of proteinase/peptidase activities have been found in T. rhodesience, some like the sulfhydryl proteinase and carboxypeptidase are predominantly associated with lysosomes, with a much smaller fraction (~30%) being associated with flagellar pocket. An even more exclusive lysosomal association was exhibited by activity toward n-t-Boc-alanyl-p-nitroanilide, originally of interest since this is a substrate used for elastase. Subsequent study using the more specific elastase substrate succ-$(Ala)_3$-p-nitroanilide failed to substantiate this enzyme as being an authentic elastase.

2) Subcellular Distribution of Antigens

a) FRI profiles for T. rhodesiense subcellular fractions. The fractions recovered after gradient isopycnic centrifugation were subjected to FRI as shown in Figure 1, using various detergent extractions and antibodies isolated from anti-P_a and anti-P_b sera. The principal lines of reaction are found between density increments 1.10 to 1.15 and 1.20 to 1.22. Within the low-density increments, eight antigenic components could be discerned, Figure 1B, whereas at least four separate antigens were resolved within the high-density region (Fig. 1A,D). Use of antibodies derived from anti-P_a sera reacted preferentially with high-density antigens (Fig. 1A) whereas anti-P_b antibodies were more reactive with low density particle antigens (Fig. 1B).

Although this basic pattern of precipitation was seen in all experiments, differences were seen depending on the detergent treatment employed. The use of 0.5% Lubrol (Fig. 1D) caused a smearing of certain lines, but appeared to selectively remove one of the low-density antigens (a) and also a slightly higher density component Ib, ρ =1.143). There was evidence of cross-reactivity between this antigen and one of the low-density particle-associated antigens. A consistent feature of 0.5% Triton X-100-extracted fractions were two precipitin peaks seen at the bottom of the gradient (b, ρ =1.24) although only using anti-P_b IgG. The zwitterionic detergent, Zwittergent 3-12 (0.4% w/v) resulted in an intense precipitin line showing little migration from the antigen wells into

the antibody-containing gel (figure not shown), presumably due to increased cathodic movement of IgG. Even lower levels (0.2%) in the presence of a nonionic detergent (0.4% Lubrol W) reduced the migration of the precipitin lines with a consequent loss of resolution. The effect was less pronounced using 0.1% Zwittergent in the presence of Triton X-100 (0.5%) Fig. 1E with good extraction of light density antigens.

Very low levels of Zwittergent 3-12 (0.1% in the presence of 0.2% Triton X-100) did appear very effective in releasing Con A-binding components, as demonstrated in Figure 1C. The most intense reaction was associated with particles in the 1.20 to 1.225 density range, with much lower density particles, densities 1.105 to 1.120, also reacting. The intensity of the reaction obscured the number of separate components involved for which crossed immunoelectrophoresis was required (see Fig. 2).

By using a split gel with lectin and anti-P_b IgG, as in Figure 1F, it is apparent that most of the low-density antigens have been absorbed by the Con A. Certain of the high-density antigens are still present, as revealed by the resulting precipitin peaks. Present in all experiments is a broad precipitin peak between densities 1.130 and 1.200, maximum reactivity being in those fractions in the middle of the density range. This antigen also appears to be reactive toward Con A, based on the lack of any corresponding precipitin peak in Figure 1F.

The use of the subcellular markers established above, permitted the unequivocal identification of the principal antigenic sites. The prominent range of antigens present in fractions from the high density end of the gradient (ρ =1.22) were undoubtedly associated with particles of surface membrane origin. The other important antigenic site is clearly the flagellar pocket membrane (FPM) which was established as equilibrating at the low density end of the gradient (ρ =1.12).

The only intracellular structures which exhibited any pronounced antigenicity were identified as lysosomes, from the subcellular markers equilibrating in that part of the

gradient.

b) Crossed and tandem-crossed immunoelectrophoresis. The low- and high-density particle-associated antigens were further resolved using crossed immunoelectrophoresis. Figure 2A reveals at least 10 antigens present in the pooled high-density fractions (from 1.195 to 1.220). The more prominent of these are designated antigens a-g. Antigens a-c all show little migration, and at least two of these, c and c', appear identical. Antigen b exhibits a prominent shoulder and is cross-reactive with d. The low-density fractions (from 1.095 to 1.125), using the same extraction (0.5% Triton X-100), exhibited at least five antigens (Figure 2B), designated v-z, with antigen x being present as a doublet. In the presence of low concentrations of Zwittergent 3-12 (0.15%), separation was improved (Figure 2C) and at least seven precipitin peaks formed, with component y being especially prominent. It should be noted that there is no experimental basis as yet to correlate the antigens extacted using the different detergent treatments. It is noticeable that little evidence can be seen from the morphology of the precipitin lines for extensive proteolytic degradation of antigens using previously established criteria (28). The split line on the righthand foot of peak w (Fig. 2C) indicated limited proteolysis.

The use of crossed-tandem electrophoresis (Figure 2D) revealed no obvious cross-reaction between antigens occurring in those two particle populations. Possibly merging of the precipitin lines x and b is discernible, indicating some cross-reactivity, but it is not clear.

c) Crossed affini-immunoelectrophoresis. Figure 3A,D shows the results obtained using crossed affini-immunoelectrophoresis with an intermediate gel containing Con A. The apparent efficacy of Zwittergent 3-12 in solubilizing lectin-binding components was previously demonstrated (Fig. 1E). At least four to five separate components were identifiable as being precipitated in the Con A gels for both samples. Two of these components showed identical migrations (La and Lb) for both low- and high-density

particles. As was indicated in Figure 1H, where most of low density antigens were removed by Con A, the effect of the lectin was now more clearly obvious (Fig. 3B). The high-density fractions still retained at least six IgG precipitable components after lectin adsorption (Fig. 3A).

d) Immunoisoelectric focusing. In Figure 4A,B the results of separating the pooled high- and low-density fractions by isoelectric focusing with electrophoresis into gels containing anti-P_a and anti-P_b IgG, respectively, are shown. Although resolution was not satisfactory, it is quite obvious that the major antigenic components of the low- and high-density fractions have quite different isoelectric points. The high-density fractions show a faint precipitin peak in the same part of the pH gradient as the major low-density component.

The major low-density precipitin peak had an isoelectric point (pI) ~5.8, whereas the high-density fractions contained a range of poorly resolved antigens with pI ~7.1. Only minor antigens migrated to higher pH values.

3) Characterization of FPM antigens

Further studies were undertaken to characterize FPM antigens, in particular the nature of the membrane association. For this purpose an FPM fraction was prepared using differential and discontinuous sucrose gradient centrifugation, as referenced in the Methods. A discussion of the distribution of marker enzymes between FPM and other subcellular fractions will be found in the reference cited (6). Here also, the use of phospholipases to study the role of lipid in binding FPM antigens and the necessary precautions in interpreting results is discussed.

a) CIEP of FPM antigens. After the FPM fraction was solubilized with 0.4% Triton X-100-0.1% Zwittergent 3-12, analysis by crossed immunoelectrophoresis produced the antigenic profile seen in Fig. 5a, which is representative of repeated experiments. Precipitin peaks a through e were all constant features, especially a, c, and e, with f and

g being more variable in occurrence. The profile obtained after this detergent extraction was used as a reference to compare other treatments of the FPM. Figure 5b shows the much reduced release of antigens after overnight extraction of FPM in hypotonic buffer (as used in Table 2, reference 6). No increase in the number or intensity of the precipitin peaks was observed after three repeated freeze-thaw cycles of the FPM fraction (not shown). Noticeable are the clear-cut indications of proteolytic degradation (28) of antigens b (flying precipitin line) and c (now present as a doublet) absent from the detergent extract. Possibly the reduced intensity of the precipitin lines, especially that of band c, is due to a loss of antigenic sequences.

b) Release of FPM antigens after exposure to phospholipase. Exposure of hypotonic buffer-washed FPM to phospholipase A_2 resulted in the release of antigen a, with an especially conspicuous precipitin peak corresponding to antigen d. Measurement of peak area d (for Fig. 5a as compared with Fig. 5c) by planimetry revealed a 17-fold increase in antigen d after detergent extraction. There was no indication of substantial release of any other antigenic components other than a and d.

Phospholipase C exposure (Fig. 5d) also caused the release of antigen a, with much less removal of antigen d. A number of other less prominent antigens (A^1 through A^3), which were not immediately comparable with detergent-released components, were also in evidence.

c) Trypsin treatment of FPM and antigen release. Trypsin treatment of FPM (Fig. 6a) caused the release of a reactive fragment of antigen a (note the incomplete precipitin peak and increased mobility) and what appeared to be a poorly reactive peak d. Subsequent phospholipase exposure of trypsin-treated FPM (Fig. 6b and c), particularly phospholipase A_2, resulted in the appearance of antigen d. Pretreatment with trypsin did not cause an increase in the amount of antigen d released, but the reduced intensity of the precipitin peak suggests that trypsin was cleaving exposed antigenic groups.

d) Separation of hydrophobic FPM antigens using Triton X-114. Phase separation of Triton X-114 was used in an attempt to partition the predominantly hydrophobic from the more hydrophilic FPM antigens. From Fig. 7a it is evident that a range of less hydrophobic antigens were recovered in the upper phase, including an incomplete peak that appears to be synonymous with antigen a (Fig. 5). For both the upper and lower phases, it was impossible to precisely correlate the antigenic profiles with those seen in Fig. 5a. However, a most conspiculous feature of the lower-phase (hydrophobic) antigen profile (Fig. 7b) was the presence of peak a' and cross-reacting antigens a^2 and a^3, none of which were present in the upper phase or discernible after Zwittergent-Triton extraction (Fig. 7a). Antigen a was also present in the lower phase and partially cross-reacted with a'.

e) Use of a lectin containing gel to demonstrate carbohydrate containing FPM antigens.

Inclusion of an intermediate gel containing concanavalin A (Fig. 8a) removed both antigens a and d released by the action of either phospholipase A_2 or C. Some minor antigens released by phospholipase C were not affected. When trypsin-pretreated FPM was exposed to phospholipase A_2, there was less retardation of antigen d by concanavalin A (Fig. 8c). This indicates that although exposure to trypsin apparently removed glycopeptide, it did not appear to affect antigenicity (compare precipitin peak d of Fig. 8c with that of Fig. 6b).

The presence of a concanavalin A intermediate gel (Fig. 8d) caused the removal of hydrophobic antigens a, a', and a^2 but not antigen a^3, which suggests that the latter is a nonglycosylated form of a' and a^2. It is not known whether these cross-reacting antigens are authentic FPM components or the products of proteolytic degradation during incubation with Triton X-114. In the upper phase, antigen c' was the only component not reactive toward concanavalin A.

f) Analysis of FPM antigens with FPM antibodies using immunoelectrophoresis and

chromatofocussing. From the above investigation, using antibodies derived from anti-sera raised against fraction P_b, it was evident that 7-8 separate antigens could be discerned after CIEP. Subsequently anti-sera obtained from rabbits repeatedly injected with FPM (see Methods) was used to more precisely characterize FPM antigens by CIEP and SDS-PAGE/immunoblotting as described (20) where 2 prominent variant stable glycoprotein antigens were identified.

4) The subcellular localization of protective antigens

The results of using fractions obtained after iopycnic gradient centrifugation of fractions Pa and Pb, as immunogens clearly reveal the presence of protective antigens against both cloned (CT clone D34) and uncloned T. rhodesiense (20). As was partly expected, fractions from the high density end of the gradient obtained after centrifuging fraction P_b and containing surface membrane fragments, confered protection. Thus for fractions 18-33 there were 19 survivors out of the 25 animals to which antigen was administered, whereas for these same fractions recovered after centrifuging fraction P_b, which contain much less surface membrane, only 12 survivors were found.

Of more interest were the results obtained using fractions recovered from the low density end of the gradients. After centrifugation of fraction P_b gradient fractions 3-6, containing flagella pocket membrane, permitted the survival of 13 out of 20 animals 28 days after challenge. Fractions in this same density range obtained after gradient centrifugation of fraction P_a, which were not enriched in FPM, gave a much lower survival rate (only 4 out of 20 animals still alive).

Other gradient fractions, corresponding to other subcellular components such as lysosomes or mitochondria have not revealed consistent protection. The most recent results obtained in conjunction with Dr. John G. Olenick at WRAIR have confirmed the immunoprophylactic nature of FPM antigen(s). Protection was afforded against challenge with both uncloned and two cloned isolates of the CT Wellcome strain of T. rhodesiense.

In this series of experiments FPM prepared by discontinuous gradient centrifugation of fraction P_b was used to inject mice prior to challenge. The results obtained are shown in Table 2.

5) The binding site on intact T. rhodesiense for FPM antibodies as revealed by immunogold electronmicroscopy.

Samples of FPM anti-sera were shipped from Miami to Dr. John Olenick (WRAIR). In conjunction with Dr. K. Nauman (University of Maryland) whole T. rhodesiense were incubated with FPM antibodies and binding sites revealed by incubation with gold conjugated goat anti rabbit IgG and examination by electron microscopy. As can be seen from Figs. 9a and b FPM antibodies react specifically with the posterior end of trypanosome at the point of insertion of the flagella. Controls show trypanosomes incubated with homologous anti-VSG antibodies (Fig. 9c) and normal rabbit serum (Fig. 10d). Note the much more generalized reaction over the entire surface of the trypanosome in the former instance as compared to the much more specific localization when FPM antibodies are used.

These results elegantly confirm the earlier cell fractionation studies concerning the derivation of the FPM fraction, and the specificity of FPM antibodies.

D. DISCUSSION AND CONCLUSIONS

Since the initial studies aimed at characterizing VSG (see 3 for review) subsequent studies have all but neglected the antigenicity and immunogenicity of other subcellular sites. The work described in this final report, and associate publications (5,6,12) represents the only comprehensive attempt so far to determine the subcellular distribution and nature of the principal antigens in any of the African trypanosomes. A review of efforts in this area prior to 1982 will be found in reference 5; since that time there have been reports on surface antigens in procyclic T. brucei (29) and attempts to ascertain the number of non-variant antigens in a number of trypanosome isolates (30).

Before considering the implications of the findings on the distribution of antigens in T. rhodesiense, it is worth commenting on the significance of some of the other findings of this study. Apart from the studies of Opperdoes and coworkers (e.g. 31) this is the only in depth study detailing the biochemical cytology of an African trypanosome and reveals much new information on their functional morphology. This is particularly so with reference to the glycosome (26), where a hypothetical model of glycosomal organization has been proposed, and the surface membrane. In the latter instance a unique high affinity Ca^{2+} ATPase was discovered as well as the unexpected presence of a tartrate resistant acid phosphatase. It will require further studies to determine the role of this surface membrane ATPase, particularly its role in regulating intracellular Ca^{2+} concentration, but this could prove an intriguing target for future chemotherapy. It is of interest that intracellular Ca^{2+} may well play a role in regulating the release mechanism for membrane bound VSG (32).

The cell fractionation studies clearly reveal the principal antigenic sites to be associated with both surface membrane and flagellar pocket membrane (FPM). Subsequent efforts have been directed toward further characterising the FPM antigens. Although it is possible that other surface membrane antigens may be partially exposed, there were good reasons for concentrating on the FPM as a target for antibody action. The flagellar pocket is a specialized invagination of the surface membrane, conspicuous at the ultrastructural level in view of the absence of an associated microtubular network (cf - surface membrane). The results of both the present investigation (5) and others have established the presence of a range of lytic enzymes, especially phosphatases and peptidases, associated with FPM suggesting a role in pre-digestion and nutrient uptake. In addition the pinocytotic uptake of macromolecules across the FPM has been demonstrated using ferritin labelled serum albumin (33) and would appear to be the route by which Triton WR was accumulated in T. brucei lysosomes (34). This probable role in

nutrient uptake appears to be specific for bloodstream forms of the parasite as opposed to procyclics (25). Such a function points to the FPM as being suitable for targeting by antibodies that may well interfere with these uptake/predigestion processes.

The above macromolecular uptake studies (33,34) also indicate that any circulating antibody recognizing specific FPM antigens would be able to gain access to the lumen of the flagellar pocket and be able to interact with externally exposed membrane components. It is probable that during actual trypanosomiasis there is minimal synthesis of antibodies to FPM antigens due to the impairment of host response resulting from overall immunosuppression (35) and the profound non-specific stimulation of polyclonal B cells by VSG (36). If purified FPM or discrete FPM antigens were to be used the latter at least would not be of concern and synthesis of any protective antibodies to FPM antigens should then be sufficient to reach levels capable of controlling a challenge infection. As discussed below, results obtained to date lend support to this proposition.

So far there is no evidence of any cross reactivity between FPM and surface membrane antigens, at least the two principal FPM antigens (20) described in this report. Both of these antigens are glycoprotein with similar M_r ($\sim$ 55-65x10^3) and intimately membrane associated. For one component there is circumstancial evidence for covalently bound lipid. This has now been well established as a feature of the mode of attachment of VSG to the surface membranes. Certainly one FPM antigen in particularly can be readily released after exposure of membrane to phospholipase A or sphingomyelinase (20). Despite any superficial similarity with VSG, the FPM antigens differ in at least two very important respects; they exhibit no obvious cross-reactivity with VSG and appear to be antigenically stable (20). Although only a limited number of cloned T. rhodesiense isolates are available, electroblots of FPM antigens after separation by SDS-PAGE exhibit no reactivity with monoclonal antibodies to VSG from isolates CP3B4 and V13 (antibodies provided by K. Esser, WRAIR). In a study where the distribution of

VSG epitopes were mapped (37), it was found that 3 out of a panel of 30 monoclonal antibodies raised to the WRAT at 1 VSG, specifically reacted with the flagellar pocket region and were capable of neutralizing infections. The resolving power of the immunofluorescence technique used could not of course permit an unequivocable statement as to the exact site of reaction. However, it is quite possible that antigenically similar sequences are present in both VSG and FPM antigens that remain to be detected.

Previous attempts to identify protective antigens other than VSG, as mentioned previously, have been extremely limited. A surface membrane component of M_r $83x10^3$, found to be variant stable in both T. brucei and T. vivax, was used in an attempt to induce protection against tsetse transmitted disease but was found to be without effect (38). Whilst it was acknowledged that this, or any other surfaces membrane component would likely not be accessible for LS forms, it was postulated that this may not be so for metacyclics introduced immediately after tsetse feeding. Hope has been expressed that the limited number of metacyclic variant types might make feasible a vaccine aimed at this stage. However, it would only need the presence of one unexpected variant type to abrogate any protection. Of special concern in T. rhodesiense, is the rapid development of metacyclic variant types that occurs (39).

The apparent variant stable and protective nature of FPM antigens (two principal antigens have been demonstrated -- see Appendix II) warrants further study. The recent use of a high resolving power two dimensional electrophoresis system to compare the migration of total proteins for various African trypanosome isolates (40) is pertinent in this respect. The only component for which there was any evidence of heterogeneity was VSG, supporting the present finding of the stability of FPM antigens.

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It is a pleasure to thank Dr. John G. Olenick (WRAIR) for his helpful criticisms and co-operation during the period of this research. Dr. Arba Ager Jr. and staff at the Rane

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E. FIGURE LEGENDS AND TABLES

1) Figure Legends

Figure 1. Fused rocket immunoelectrophoresis of fractions obtained after isopycnic centrifugation (see 4,5) of a T. rhodesiense high speed pellet. A, B, both fractions and gel contained 0.5% Triton X-100. C, samples contained 0.15% Zwittergent 3-12 and electrophoresed into gel containing 80 g/cm^2 Concanavalin A and 0.15% Zwittergent. D, samples and gel contained 0.4% Lubrol W. E, fractions and gel contained 0.5% Triton X-100 and 0.1% Zwittergent 3-12. F, using 0.5% Triton X-100. The following antibodies were used: gels A, D contained anti-P_aIgG; B, E contained anti-P_bIgG.

Figure 2. Crossed and tandem-crossed immunoelectrophoresis of pooled fractions recovered after isopycnic centrifugation. A, pooled high-density fractions (= 1.195-1.22) B, pooled low-density fractions, both extracted with 0.5% Triton X-100 with antibody gel containing 0.5% Triton X-100 and anti-P_b IgG. C, pooled low-density fractions using 0.15% Zwittergent 3-12 in the first dimension and 0.1% Zwittergent/0.4% Triton X-100 for second dimension electrophoresis with a gel containing anti-P_b IgG. D, tandemcrossed immunoelectrophoresis, first slot containing pooled high-density fractions and second slot pooled low-density fractions. Detergent and antibodies used as for A and B.

Figure 3. Crossed affini-immunoelectrophoresis of pooled high-density fractions A and pooled low-density fractions B. Electrophoresis conditions as for Figure 4C. Upper gels contained anti-P_b IgG, both lower gels contained Concanavalin A (80 g/cm).

Figure 4. Immuno-isoelectric focusing of pooled high-density A and low-density fractions B using a pH 5.0-8.5 ampholyte gradient. Bars indicate 0.5 pH unit.

Figure 5. Crossed immunoelectrophoresis of a T. rhodesiense FPM fraction. Components released after (a) extraction with 0.1% Zwittergent 3-12-0.4% Triton X-100, (b) overnight extraction at 0°C in 5mM calcium acetate-20 mM HEPES (pH 7.5), and (c and d) exposure

of FPM, after extraction b, to phospholipases A_2 and C, respectively. In all instances, an antibody-containing agarose gel was used that contained anti-P_b immunoglobulin G (5) at a final concentration of 7.5%

Figure 6. Crossed immunoelectrophoresis of a T. rhodesiense FPM fraction after mild trypsinization. Precipitin patterns obtained for material released after (a) trypsinization alone, (b) trypsinization followed by exposure to phospholipase A_2 and (c) trypsinization followed by exposure to phospholipase C. Agarose gel contained detergent and anti-P_b immunoglobulin G as for Fig. 5.

Figure 7. Antigenic profiles of T. rhodesiense FPM hydrophilic and more hydrophobic membrane components partitioned by extraction with Triton X-114 (a) crossed immuno-electrophoresis of hydrophilic membrane components recovered in the upper bulk aqueous phase after Triton X-114 extraction (b) crossed immunoelectrophoresis of more hydro-phobic membrane components partitioned into the detergent enriched power phase. Detergent and antibody incorporated as previously described for Fig. 5.

Figure 8. Crossed affini-immunoelectrophoresis of a T. rhodesiense FPM fraction with an intermediate gel containing concanavalin A (Con A). Precipitin patterns obtained for components released after (a) (b) exposure to phospholipases A_2 and C respectively, and (c) mild trypsinization followed by exposure of residual FPM to phospholipase A_2. (c) As for Fig. 7b, showing removal of Con A reactive components from lower phase after Triton X-114 extraction of FPM. Intermediate lectin containing gel prepared using 80 g of Con A per cm^2. Detergent and antibody as for Fig. 5.

Figure 9. Binding site of FPM antibodies as demonstrated by immunogold electronmi-croscopy. (a) With FPM as primary antibody showing highly localized reaction site where flagellum enters posterior end of the trypanosome. (b) As for (a) but showing increased magnification of posterior end of organism. (c) For comparison notice generalized binding of immunogold using anti-VSG as primary antibody. (d) Control using normal

rabbit serum as primary antibody.

The FPM antibodies were raised in New Zealand white rabbits at U.M. and sent to WRAIR where immunogold electronmicroscopy was performed by Dr. John G. Olenick and Dr. Robert Nauman (U. Maryland).

Subcellular Site (equilibrium density)	Enzyme Activity Present
Flagellar Pocket Membrane (ρ = 1 180-1 120g/cm^3	Acid phosphatase[1] (both tartrate resistant and sensitive activities* are present) Various peptidases (* leucyl raphthyl amidase, leucine amino peptidase[2] most specific), Co^{2+} dependent 3'-nucleotidase
Mitochondrion (ρ = 1 140g/cm^3	DCPIP linked -glycerophosphate dehydrogenase*. Some of ATPase and "GTPase" activity[3] with minor amounts of Ca-ATPase and adenylate kinase
Lysosome (ρ = 1 170-1 175g/cm^3	Most of the thiol proteinase*, carboxypeptidase*, and "elastase" like enzyme[4]. Lesser proportions of leucyl naphlhylamidase and leucine amino-peptidase[2] activities
Surface Membrane (ρ = 1 220g/cm^3	Ca^{2+} ATPase, approximately half the tartrate resistant acid phosphatase, a portion of the leucine amimo peptidase
Glycosome (ρ = 1 230g/cm^3	Core: NAD^+ linked -glycerophosphate dehydrogenase*, hexokinase, phosphofractokinase membrane: adenylate kinase

Table 1. Summary of enzyme distribution in T. rhodesiense information is based on previously published reports (2, 4, 5, 25,) and Appendix I.

1 - Distribution profiles and properties of acid phosphatase activities will be found in reference 12 - note that whilst most of tartrate sensitive acid phosphatase is exclusively associated with flagellar pocket, the tartrate resistant phosphatase is found in both surface membrane and flagellar pocket membrane 2 - Activity toward leucinamide is not due to an authentic Co^{2+}/Mn^2 stimulated leucine amino peptidase, a range of such metal dependent aminopeptidases are present in the cytosol of T. rhodesiense. 3 - GTPase activity probably due to mitrochondrial ATPase - see text. 4 - Not a true elastase - see text concerning lysosomes. * Routinely used as marker enzymes. For surface membrane fluorescamine labelling is used as a marker.

Immunization Received	Trypanosome Isolate for challenge	Number of mice per group	Cumulative Numbers of mice dead on days										Percent Survival
			5	6	7	8	9	10	11	12	15	30	
FPM + FCP	CP384	5	-	-	2	-	-	-	-	-	2	2	60
	Y10	5	-	-	-	-	1	2	-	3	3	3	40
	Y13	5	-	1	5	-	-	-	-	-	5	5	6
FCP+ Water	CP384	5	2	4	5	-	-	-	-	-	5	5	0
	Y10	5	-	3	4	5	-	-	-	-	-	-	0
	Y13	5	-	-	4	-	5	-	-	-	5	5	0

Table 2. Cross protection of mice induced by FPM fraction on challenge with three variant antigenic types of Trypanosoma rhodesiense. The T. rhodesiense FPM fraction was obtained at U.M. and dispatched on dry ice to Dr. John G. Olenick (WRAIR) who performed the animal trials shown.

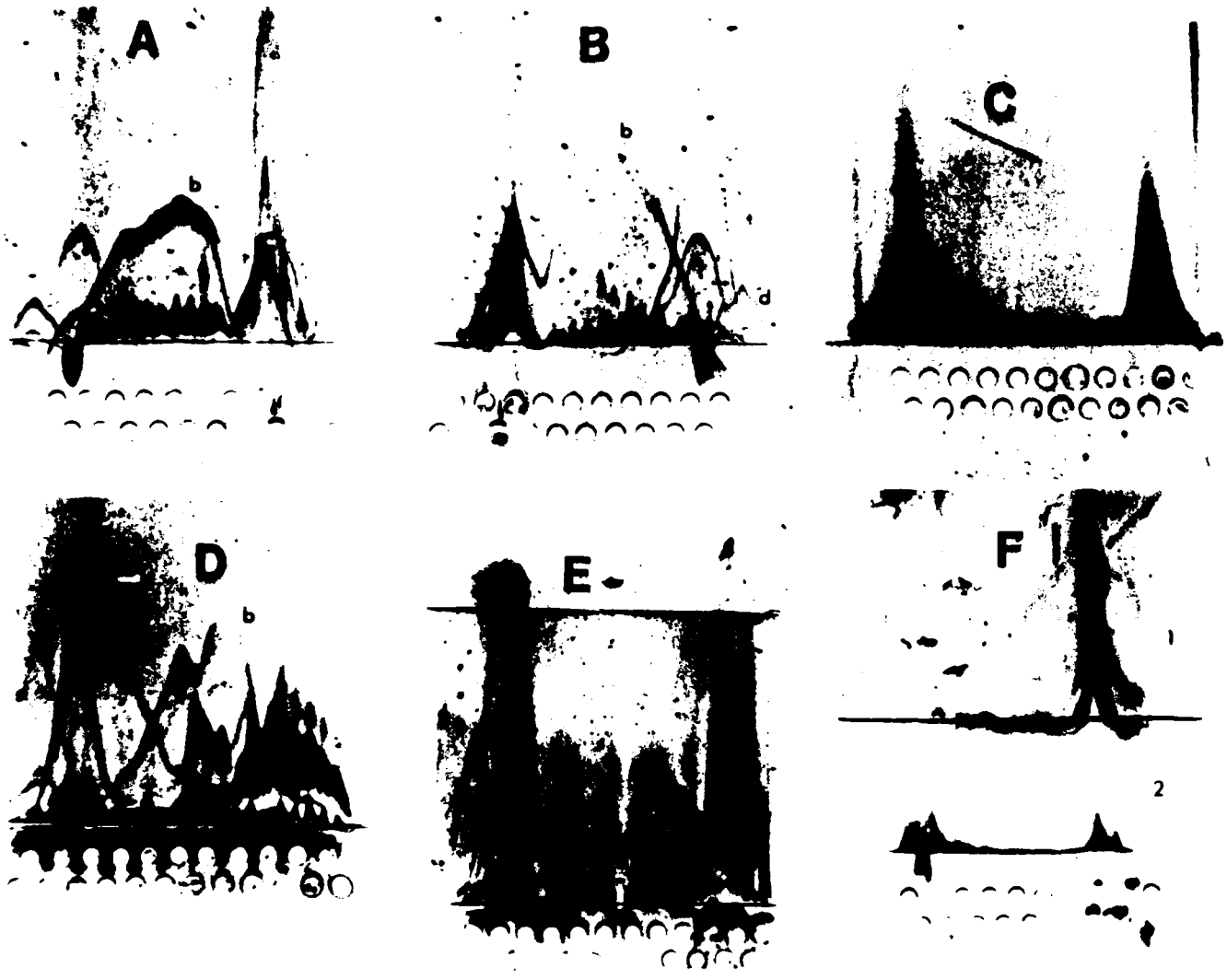

FIG. 1

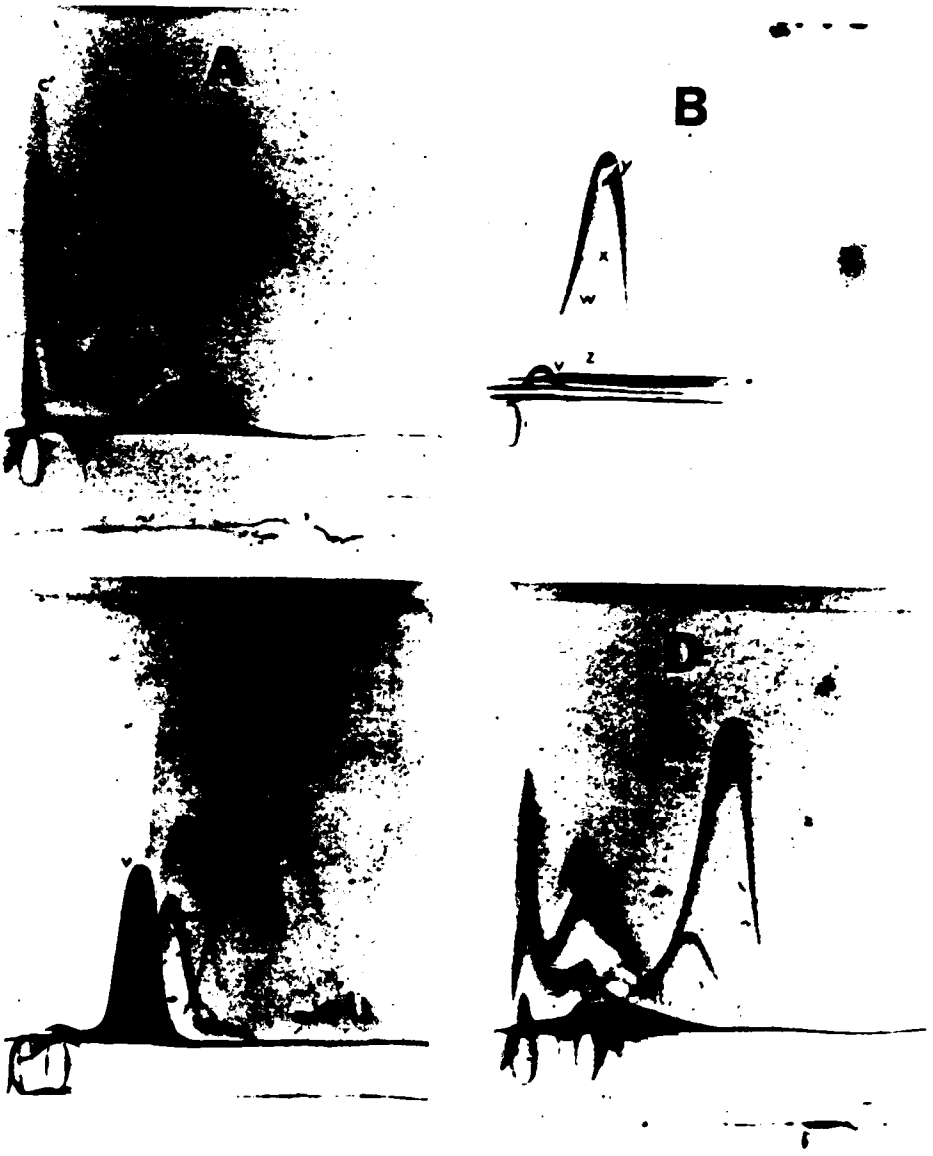

FIG. 2

FIG. 3

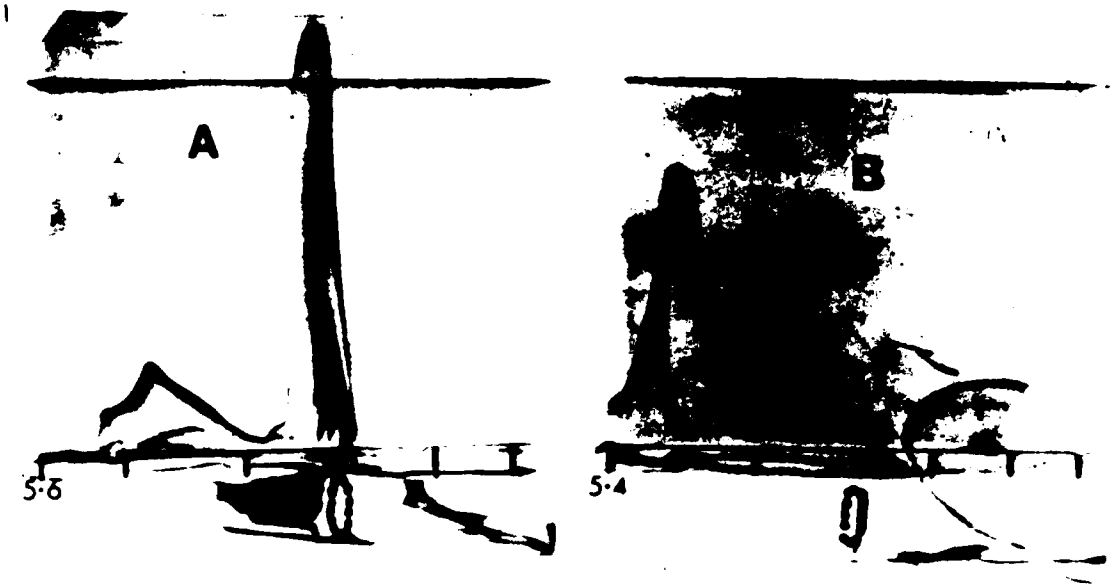

FIG. 4

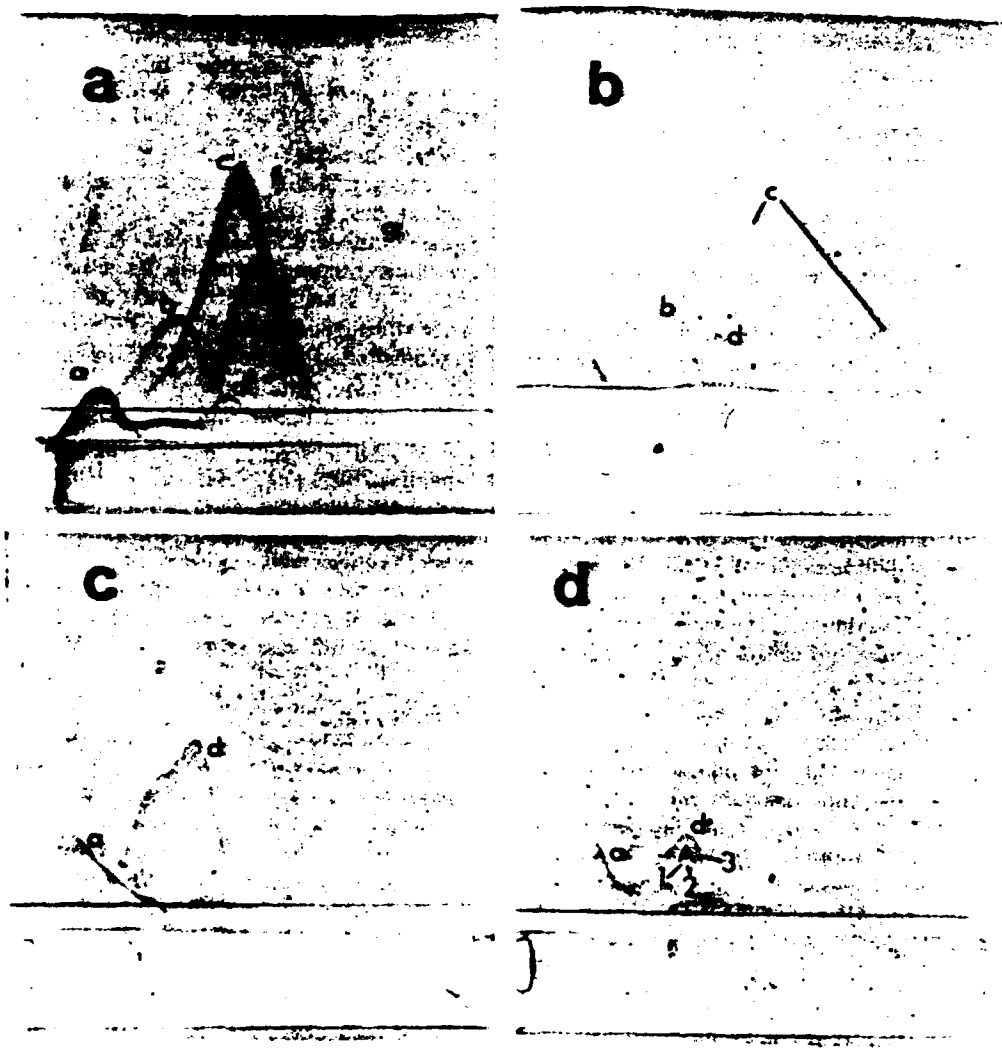

FIG.5

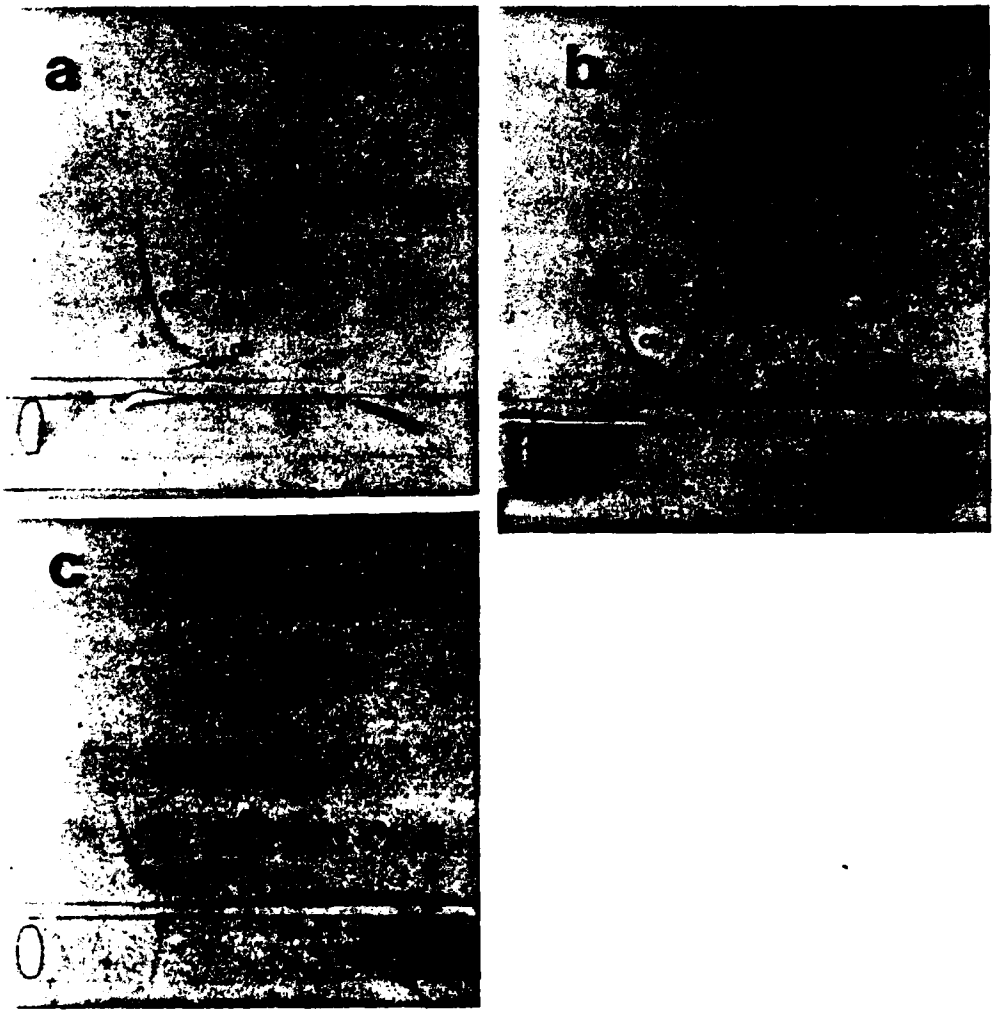

FIG. 6

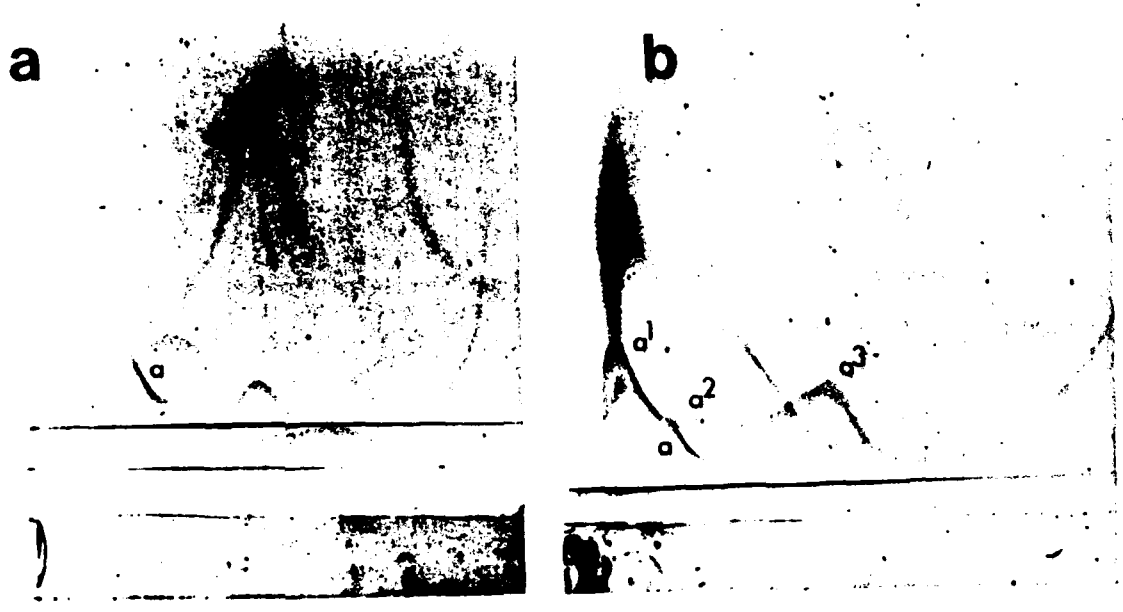

FIG. 7

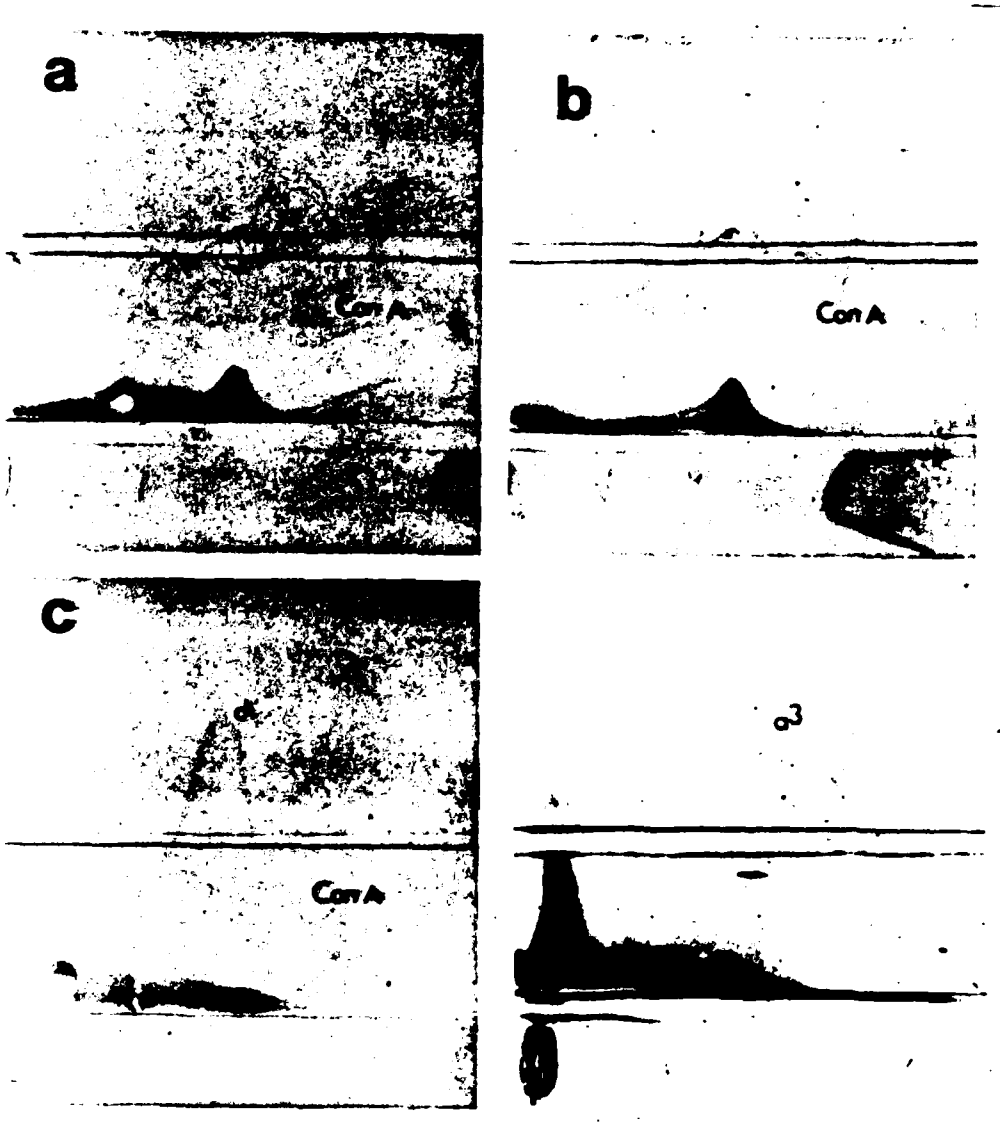

FIG. 8

anti-FPM as primary antibody
Note: highly specific labeling of
invaginated area of entry of
flagellum into cell

FIG 9(a,b)

anti-surface coat as primary antibody
Note: scattered, non-specific labeling
of immunogold

FIG 9(c,d)

END

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